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Evaluation of automated direct sample introduction with comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry for the screening analysis of dioxins in fish oil

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ABSTRACT

An automated direct sample introduction technique coupled to comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (DSI-GC × GC/TOF-MS) was applied for the development of a relatively fast and easy analytical screening method for 17 polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/Fs) and 4 non-ortho polychlorinated biphenyls (PCBs) in fish oil. Comparison of instrumental performance between DSI-GC × GC/TOF-MS and the traditional gas chromatographic high resolution mass spectrometric (GC-HRMS) method showed good agreement of results for standard solutions analyzed in blind fashion. Relatively high tolerance of the DSI technique for lipids in the final extracts enabled a streamlined sample preparation procedure that only required gel permeation chromatography (GPC) and solid-phase extraction (SPE) cleanup with graphitized carbon black. The sample size for the method was 2 g of cod liver oil, which achieved limits of quantitation (LOQs) of 0.019–7.8 pg/g toxic equivalent quotients for the individual PCDD/Fs. Lower detection limits can be achieved by using larger sample size and scaling up the sample preparation procedure, but this adds to the labor, time, solvent consumption, and expense of the approach. However, the streamlined method yielded 0.94 pg/g and 2.3 pg/g LOQs for 2,3,7,8-tetrachloro dibenzofuran (TCDF) and 3,3′,4,4′,5-pentachloro biphenyl (CB126), which were sufficiently low for regulatory monitoring of 2 g samples. Therefore, instead of congener specific analysis, this streamlined analytical screening method for TCDF and CB126 has the potential to monitor fish oil contaminated with dioxin and dioxin-like PCBs at or above current food safety limits. Acceptable recoveries for nearly all analytes at three different spiking levels in fish oil samples were achieved with good repeatability.

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1. Introduction

The 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are well-known toxic contaminants, and humans are exposed to these toxic chemicals mostly through food consumption [1]. Past incidents of dioxin crises in the food chain have occurred in Belgium and the USA in the 1990s [2–4]. Following the crises, the EU established a food safety regulation for the 17 PCDD/Fs and dioxin-like polychlorinated biphenyls (PCBs) [5] and in the USA, the US Department of Agriculture's Food Safety Inspection Service and US Food and Drug Administration set a temporary action level of 1 pg/g (ppt) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [6].

The food safety regulations require routine monitoring of dioxins in many samples of foodstuffs, but the most common analytical method used worldwide for dioxin analysis, which is based on high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS) [6], is very time consuming and expensive. The method works exceptionally well for congener specific dioxin analysis at extremely low levels, which is good for risk assessment purposes, but it is not well-suited for regulatory monitoring and screening analysis. Therefore, several alternate analytical approaches have been investigated for dioxin analysis to develop less costly and more efficient analytical methods [7–12]. However, these alternate methods still require thorough clean-up procedures based on several adsorptive columns for fatty and other complex matrices. One of the reasons for such extensive cleanup pertains to the typical use of splitless injection in GC, which lacks ruggedness for relatively dirty matrices. Screening methods based on enzyme immunoassays or other bioassays have been introduced [13,14], but they also require extensive clean-up, especially lipid removal.

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Moreover, these types of assays are not quantitative, nor are they able to qualitatively identify the analytes or give congener profiles [6].

Large-volume injection (LVI) is an increasingly useful tool in GC to achieve simpler sample preparation, smaller sample size, and/or better sensitivity [15]. However, there are only few investigators who reported employing LVI for dioxin analysis [16,17]. Even in those examples, the sample preparation still included many steps entailing adsorptive clean-up. One novel form of LVI, known as direct sample introduction (DSI), was originally invented by Amirav and colleagues [18,19] to allow LVI of dirtier samples, yet still maintain a clean inlet and column. Automated versions of DSI have since been introduced and adapted for multi-residue pesticide analysis in various food matrices, demonstrating its robustness for dirty matrix samples [20–23]. For this study, we chose to apply the automated DSI-LVI technique for dioxin analysis in an attempt to reduce sample preparation needs. For the analytical method to detect the most important 17 PCDD/Fs and 4 non-ortho PCBs, we used our previously optimized conditions of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/TOF-MS) [24]. Taking advantage of greater separation ability from GC × GC/TOF-MS and higher tolerance for dirty matrices from the DSI-LVI technique, we aimed to develop a reasonably fast and inexpensive analytical method requiring less clean-up for regulatory monitoring and screening purposes. Considering the important health benefits from fish, but global contamination with persistent organic pollutants (POPs) in fish/fish oil [25], we chose to use cod liver oil as the matrix for method development and potential future application.

GC × GC coupled with micro-electron capture detection (μECD) has also been reported as a potential lower-cost screening method for dioxins in food matrices [26,27]. GC × GC-μECD does not allow the use of isotopically labeled internal standards, which is a major benefit of isotope dilution MS for potential quantitation. Another major advantage of full scan MS analysis over μECD is the ability to identify nontargeted unknowns in the samples, as well as the dioxin analytes of interest.

2. Experimental

2.1. Materials

EPA-8290STN solution (17 PCDD/Fs), EPA-8280IS solution (mixture of $^{13}C_{12}$-PCDD/Fs, TCDD, TCDF, HxCDD II, HpCDF I and OCDD), MBP-MXF solution (4 $^{13}C_{12}$-non-ortho PCBs), and $^{13}C_{12}$-CB189 were purchased from Wellington Laboratories (Guelph, Canada). The codes for the individual PCDD/Fs and PCBs are described in Table 3, in which T signifies tetra, Pe is for penta, Hx represents hexa, Hp denotes hepta, and O stands for octa. Four native non-ortho PCBs were purchased individually from AccuStandard (New Haven, CT, USA).

Two bottles of the same brand of cod liver oil were purchased from an internet retailer; Bottle A had a label indicating "PCB/heavy metal free", but Bottle B did not. The peak intensities of PCBs in Bottle B were much higher than Bottle A, but this difference did not affect our experiments.

All solvents used in this study were HPLC grade; cyclohexane and toluene were purchased from Sigma–Aldrich (Milwaukee, WI, USA), and dichloromethane (DCM), hexane, ethyl acetate (EtOAc) and iso-octane were purchased from J.T. Baker (Phillipsburg, NJ, USA). For solid-phase extraction (SPE) clean-up using graphitized carbon black (GCB), disposable Supelclean Envi-Carb reversible cartridges (175 mg of GCB and 0.5 mL polypropylene reversible tube) were custom-made by Supelco (Bellefonte, PA, USA). In addition, bulk GCB and disposable polypropylene reversible tubes (0.5 mL) were purchased from Supelco. Another type of GCB, Hypersep Hypercarb (30–40 μm, 5 g), was provided by Thermo-Fisher Scientific (Warwickshire, UK).

Septa for the autosampler vial caps were heated at 200 °C overnight and all glassware were heated at 450 °C for 6 h in a muffle furnace prior to use.

2.2. Sample preparation

For lipid removal, an automated gel permeation chromatograph (GPC) (J2 Scientific, Columbia, MO, USA) was employed. The glass GPC column was 2 cm i.d. and 22.5 cm length packed with 24 g of BioBeads S-X3 in 1:1 EtOAc:cyclohexane (v:v) and purchased from J2 Scientific. Prior to GPC sample injection, 4 g of cod liver oil was spiked with known amounts of isotope-labeled internal standard solution containing 5 $^{13}C_{12}$-PCDD/Fs (TCDF, TCDD, HxCDD II, HpCDF I, and OCDD) and 4 individual non-ortho $^{13}C_{12}$-PCBs and was dissolved in mobile phase solvent (1:1 EtOAc:cyclohexane) taken up to 10 mL in a volumetric flask. Half (5 mL) of the solution was injected into the GPC with flow rate set at 5 mL/min. The eluent fraction between 12.5 min and 17.5 min (~25 mL) was collected in a 30 mL glass tube, and the GPC extract was transferred manually to a glass syringe tube attached to a reversible SPE cartridge containing GCB. The SPE flow rate was set at ~3 drops/s using a vacuum manifold. An additional 4 mL of mobile phase solvent was passed through the cartridge to wash the column of nonretained matrix components. Then, the SPE cartridge was turned upside down using polypropylene adaptors (female and male Luer couplers purchased from Supelco). For elution of the analytes, 7 mL of toluene was passed through the cartridge and collected in a 7-mL amber glass vial. The toluene extract was heated in a heating block and taken to near dryness under a stream of nitrogen. Then the extract was quantitatively transferred to an autosampler vial containing a microvial insert using multiple 100 μL rinses of iso-octane followed by further evaporation steps under nitrogen flow. $^{13}C_{12}$-CB189 was added as a quality control measure and final volume was 100 μL in iso-octane.

The determination of recovery and reproducibility was done using the cod liver oil from Bottle A fortified with three levels of standard solutions (Table 3) with three replicates each. For matrix-matched calibration standards, prepared standard solutions were added to final extracts of cod liver oil from Bottle A (blank controls) to yield the desired concentrations.

2.3. DSI-GC × GC/TOF-MS

A Pegasus 4D (Leco, St. Joseph, MI, USA) GC × GC/TOF-MS was used in this study, and the GC × GC/TOF-MS parameters had been previously optimized [24]. The conditions were slightly modified for DSI and summarized as follows (see Ref. [24] for further details): a Restek (Bellefonte, PA, USA) Siltek deactivated column (4 m, 0.25 mm i.d.) was attached to the inlet as a guard column; a Restek Rtx-Dioxin 2 (60 m, 0.25 mm i.d., 0.25 μm film thickness) served as the first dimension column (1D) and an Rtx-PCB (3 m, 0.18 mm, 0.18 μm) was the second dimension column (2D); ultra-purity helium (Airgas, Radnor, PA, USA) was used as the carrier gas; the primary oven temperature program entailed initial temperature at 60 °C for 7.5 min, ramped at 10 °C/min to 300 °C where it was held for 25 min; and the secondary oven temperature was programmed to be 20 °C higher than the primary oven. For GC × GC, the modulation period was set as 3.5 s with 0.9 s hot pulse duration and 35 °C modulator temperature offset vs. the primary oven temperature. The MS transfer line was held at 270 °C. The ion source temperature was 250 °C, the electron energy was 80 eV, the detector

voltage was 1800 V, and data acquisition rate was 50 spectra/s.

Injection was conducted by a Combi-PAL autosampler (Leap Technologies; Carrboro, NC, USA) with the automated DSI accessory (Linex) in combination with an Optic 3 programmable temperature vaporizer (Atas-GL International, Veldhoven, The Netherlands). The optimized conditions for DSI of final extracts in iso-octane were as follows (10 μL injection volume): initial injector temperature was held at 100 °C for 7.5 min with 50:1 split ratio, ramped to 300 °C at the maximum rate (16 °C/s) with splitless period of 7.5 min, then 50:1 split ratio for 16.5 min at which point the split flow was reduced to 25:1 and the injection temperature was cooled to 250 °C. The gas flow rate was held at 2 mL/min for 7.5 min (solvent evaporation time), ramped to 5 mL/min as a pressure pulse during the 7.5 min splitless period, then reduced to 2 mL/min until 35 min, ramped to 2.5 mL/min until 47 min, and taken to 3 mL/min until the end of the analysis to better elute the last analytes (OCDD and OCDF). The total analysis time was ~63 min including the time for solvent evaporation in the inlet.

2.4. Quantitation

Isotopically labeled internal standards were used for quantitation of each congener of the 4 non-ortho PCBs. The 17 PCDD/Fs were calculated against 5 $^{13}C_{12}$-PCDD/Fs: $^{13}C_{12}$-TCDF for TCDF, $^{13}C_{12}$-TCDD for TCDD and PeCDD/Fs, HxCDD II for HxCDD/Fs, $^{13}C_{12}$-HpCDF I for HpCDD/Fs, and $^{13}C_{12}$-OCDD for OCDD/F. For a recovery check of the SPE clean-up step, the recoveries of the internal standards were calculated against $^{13}C_{12}$-CB189 added to the final extracts. For the determination of recovery and repeatability, five levels of matrix-matched calibration standards were used in duplicate injections (5 pg/g, 10 pg/g, 25 pg/g, 50 pg/g, and 100 pg/g of TCDD with other congeners following the ratios shown in Table 3).

2.5. HRGC-HRMS analysis

Standards solutions in iso-octane were prepared independently at concentrations unknown to the analysts and analyzed separately by HRGC-HRMS and DSI-GC × GC/TOF-MS. Results were compared to determine the relative performances of each approach, including limits of quantitation (LOQs). HRGC-MS was performed at the ARS Biosciences Research Laboratory according to a modification of EPA Method 1613A for PCDD/Fs that included the non-ortho PCBs [28]. The standard solutions were quantitated by isotope-dilution methods using standards purchased from Wellington Laboratories (Guelph, Canada): EPA-1613CSL–4 (calibration standards for 17 native PCDD/Fs), EPA-1613LCS (15 $^{13}C_{12}$-PCDD/Fs), BP-CB (3 native non-ortho PCBs), and MP-CP (3 $^{13}C_{12}$-non-ortho PCBs). HRGC-MS conditions included 2 μL splitless injection (250 °C), separation on a J&W Scientific DB-5 ms column (60 m, 0.32 mm i.d., 0.25 μm film thickness) with a deactivated pre-column (0.5 m, 0.53 mm i.d.), and detection on an Autospec Ultima mass spectrometer (Waters Corp., Milford, MA) at a resolution of 10,000.

2.6. Statistical analysis

A commercial statistical software, The Unscrambler version 9.6 (CAMO software Inc., Woodbridge, NJ, USA) was utilized for design of experiments to optimize various DSI parameters.

3. Results and discussion

3.1. Optimization of DSI

The automated DSI (Linex) device requires several parameters to be optimized. To screen and evaluate the parameters quickly, we applied a statistical design of experiments (DOE) approach. We divided the parameters to two parts: analyte transfer efficiency and solvent ventilation. In DSI, LVI is used with a disposable glass microvial in the liner, and excessive solvent should be ventilated to reduce volume from 10 μL to 1–2 μL before transfer to the GC column. First of all, five parameters to assess transfer efficiency (flow rate, transfer time, oven temperature, initial inlet temperature, and final temperature) were screened and optimized by using full factorial design according to the conditions shown Table 1. In the experiment, 2 μL of the 17 PCDD/F standard solutions in iso-octane was added to the microvials, and GC/TOF-MS without solvent ventilation time was used for analysis. Each congener's peak area was compared by analysis of variance (ANOVA) at 95% confidence level ($P < 0.05$). The results are summarized in Table 1. Flow rate at 4–6 mL/min produced maximum peak area (transfer efficiency), transfer time ≥5 min was desirable, and higher final temperature gave maximum peak areas, especially for OCDD and OCDF. The initial oven temperature was not significant, but we observed that lower oven temperature seemed to improve focusing of TCDD and TCDF at the front of the GC column. Initial inlet temperature was not significant within the test range (80–120 °C). For the optimal conditions, we chose flow rate of 5 mL/min, transfer time of 7.5 min, initial oven temperature of 60 °C, and final inlet temperature of 300 °C (the maximum sustained temperature of the primary

Table 1
Optimization of DSI transfer efficiency for 2 μL injection of PCDD/Fs in iso-octane

Parameter	Test range	Significance[a]	Optimal condition
Flow rate (mL/min)	1–10	Yes	4–6
Transfer time (min)	1–10	Yes	≥5
Oven temperature (°C)	60–120	No[b]	60
Inlet initial temperature (°C)	80–120	No	100
Inlet final temperature (°C)	200–350	Yes	300

[a] The significance test (ANOVA) was conducted by a statistics software, Unscrambler 9.6 (CAMO).

[b] Lower temperature seems better for some PCDD/Fs.

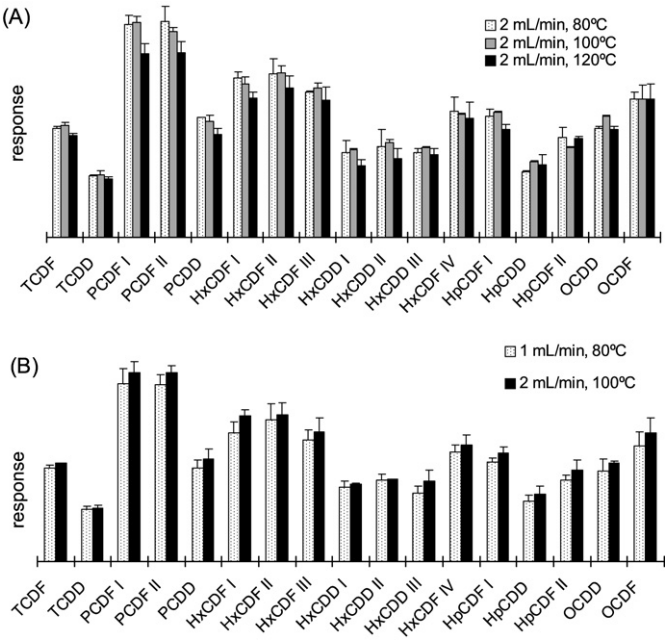


Fig. 1. Comparison of analyte responses using different solvent vent conditions: (A) different inlet temperatures (and vent times) during venting at 2 mL/min flow rate and (B) two different flow rates and initial inlet temperatures with the same vent time ($n = 3$).

column used). Since the inlet initial temperature was not significant for transfer efficiency, we optimized this parameter during the solvent ventilation experiments.

Three parameters such as solvent venting temperature (inlet initial temperature), flow rate, and split ratio were tested to determine how these factors affect solvent evaporation time. We chose to inject 10 μL for final extracts in iso-octane in the method. Thus 10 μL amounts were transferred to microvials in DSI, and its evaporation time to ~2 μL was measured at each condition, which was also generated by full factorial design as the previous test was done. The temperature range for the test, 80–120 °C, was chosen based on iso-octane's boiling point (99.3 °C). Otherwise, flow rates from 0.5 to 2 mL/min and split ratios from 25:1 to 200:1 were tested. According to the software, the temperature and flow rate were found to be significant to solvent evaporation time, whereas the split ratio was not significant. Higher temperature and lower flow rate led to shorter evaporation time, respectively.

To determine optimal solvent venting conditions, potential losses of the analytes were tested during solvent evaporation in the DSI microvial. We chose three inlet temperatures (80 °C, 100 °C, and 120 °C) with 2 mL/min flow rate and 50:1 split ratio; 10 μL of the 17 PCDD/Fs standard solution was injected to a microvial, and each congener's peak area was compared. Fig. 1(A) shows some losses of TCDF, PeCDFs and PeCDD at the higher temperature (120 °C). There is not much difference between the other two temperatures, and since 100 °C is favorable to 80 °C due to its shorter evaporation time (7.5 min vs. 17 min), it was given preference. We compared this condition with 1 mL/min flow rate at 80 °C initial inlet temperature, which had similar solvent evaporation time. Fig. 1(B) shows that there is not much difference between the two conditions. Ultimately, we selected 100 °C inlet temperature, 2 mL/min flow rate, and 50:1 split ratio for the 17 PCDD/Fs.

3.2. Instrumental performance of DSI-GC × GC/TOF-MS

To verify instrumental performance of GC × GC-TOF MS with 10 μL injection using DSI conditions described above, we conducted a comparison study with HRGC-HRMS. Seven standard solutions of different levels of the 17 PCDD/Fs in iso-octane were prepared by a third-party (our co-worker) and were analyzed separately in blind fashion using DSI-GC × GC/TOF-MS in the laboratory in Wyndmoor, PA and HRGC-HRMS in the laboratory in Fargo, ND. Calibration standard solutions and internal standards were made separately in each laboratory. Table 2 summarizes the results; the values from both analytical methods agreed well with the actual added values. In addition, both methods showed good repeatability. Samples 4 and 5 were made from the same solution and the calculated concentrations for those samples in each method were very close. All the data except non-detects are plotted in Fig. 2. This showed that DSI-GC × GC/TOF-MS tended to overestimate the levels of the analytes slightly and HRGC-HRMS underestimated them slightly, but this was probably related to calibration more than the instrumental method. Overall, the calculated values from each method agreed well with the expected values. Sample 1 was a blank sample, but

Table 2
Comparison of instrumental performance of DSI-GC × GC-TOF-MS and HRGC-HRMS using blind samples prepared in iso-octane (concentrations in pg/μL)

PCDD/F	LOQ		Sample 1			Sample 2			Sample 3		
	TOF	HRMS	Added	TOF	HRMS	Added	TOF	HRMS	Added	TOF	HRMS
TCDD	<0.02	<0.02	0	<LOQ	<LOQ	0.050	0.049	0.043	0.380	0.443	0.322
PeCDD	<0.14	<0.04	0	<LOQ	0.057	0.125	<LOQ	0.123	0.950	1.080	0.790
HxCDD I	<0.12	<0.05	0	<LOQ	<LOQ	0.125	0.154	0.110	1.450	1.676	1.122
HxCDD II	<0.08	<0.05	0	<LOQ	<LOQ	0.125	0.143	0.108	0.950	1.239	0.775
HxCDD III	<0.11	<0.06	0	<LOQ	<LOQ	0.125	0.116	0.126	0.950	1.022	0.760
HpCDD	<0.17	<0.06	0	<LOQ	<LOQ	0.125	<LOQ	0.139	0.950	1.098	0.801
OCDD	<0.45	<0.09	0	<LOQ	<LOQ	0.250	<LOQ	0.246	1.900	2.279	1.644
TCDF	<0.02	<0.02	0	<LOQ	<LOQ	0.050	0.056	0.054	0.380	0.432	0.352
PeCDF I	<0.05	<0.02	0	<LOQ	0.041	0.125	0.155	0.112	0.950	1.115	0.750
PeCDF II	<0.03	<0.03	0	<LOQ	0.051	0.125	0.169	0.114	0.950	1.118	0.810
HxCDF I	<0.04	<0.03	0	<LOQ	0.038	0.125	0.098	0.126	0.950	1.033	0.762
HxCDF II	<0.05	<0.03	0	<LOQ	0.032	0.125	0.131	0.121	0.950	1.119	0.757
HxCDF III	<0.08	<0.03	0	<LOQ	0.040	0.125	0.111	0.105	0.950	1.070	0.733
HxCDF IV	<0.09	<0.04	0	<LOQ	<LOQ	0.125	0.151	0.133	0.950	1.048	0.765
HpCDF I	<0.10	<0.04	0	<LOQ	0.050	0.125	0.131	0.123	0.950	1.101	0.761
HpCDF II	<0.15	<0.05	0	<LOQ	<LOQ	0.125	<LOQ	0.122	0.950	1.115	0.752
OCDF	<0.40	<0.08	0	<LOQ	<LOQ	0.250	<LOQ	0.252	1.900	2.322	1.554

PCDD/F	Sample 4 & 5					Sample 6			Sample 7		
	Added	TOF I	TOF II	HRMS I	HRMS II	Added	TOF	HRMS	Added	TOF	HRMS
TCDD	0.350	0.342	0.384	0.267	0.258	0.837	0.828	0.635	0.930	1.026	0.673
PeCDD	0.700	0.787	0.762	0.629	0.664	0.720	0.722	0.622	0.800	0.789	0.678
HxCDD I	0.600	0.741	0.721	0.551	0.548	0.990	0.968	0.860	1.100	0.940	0.912
HxCDD II	0.800	0.741	0.853	0.593	0.624	0.810	0.868	0.631	0.900	0.813	0.678
HxCDD III	1.000	1.066	1.162	0.868	0.877	0.900	0.913	0.755	1.000	1.209	0.834
HpCDD	0.900	0.935	0.900	0.768	0.753	0.630	0.603	0.553	0.700	0.746	0.617
OCDD	0.500	0.542	0.532	0.544	0.520	0.810	0.819	0.675	0.900	0.902	0.860
TCDF	0.100	0.116	0.108	0.108	0.106	0.162	0.158	0.145	0.180	0.190	0.164
PeCDF I	0.250	0.276	0.319	0.255	0.257	0.405	0.414	0.331	0.450	0.485	0.371
PeCDF II	0.250	0.307	0.299	0.265	0.278	0.405	0.428	0.366	0.450	0.487	0.405
HxCDF I	0.250	0.303	0.296	0.243	0.255	0.405	0.356	0.345	0.450	0.505	0.385
HxCDF II	0.250	0.247	0.338	0.251	0.257	0.405	0.440	0.363	0.450	0.526	0.384
HxCDF III	0.250	0.260	0.301	0.237	0.255	0.405	0.389	0.335	0.450	0.477	0.370
HxCDF IV	0.250	0.297	0.305	0.248	0.255	0.405	0.412	0.336	0.450	0.470	0.367
HpCDF I	0.250	0.291	0.298	0.251	0.254	0.405	0.393	0.360	0.450	0.518	0.389
HpCDF II	0.250	0.257	0.235	0.256	0.245	0.405	0.373	0.363	0.450	0.522	0.393
OCDF	0.500	0.586	0.595	0.491	0.515	0.810	0.899	0.665	0.900	0.954	0.763

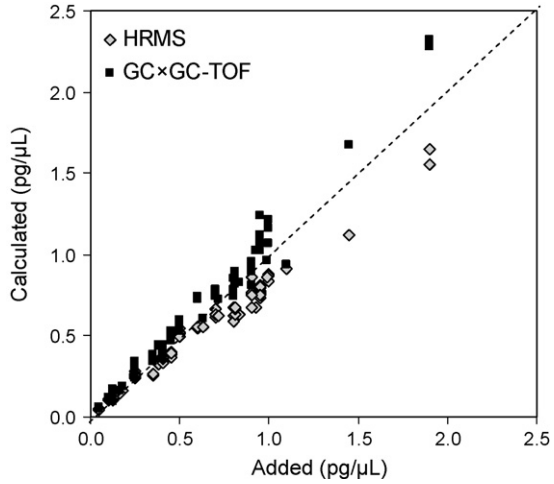


Fig. 2. Comparison of analyte concentrations obtained in blind samples (see Table 2) using HRGC-HRMS and DSI-GC × GC/TOF-MS. The dashed line represents the expected values (added concentrations).

some PCDD/Fs were detected at ultratrace levels by HRGC-HRMS, indicating slight contamination or other factor leading to the findings. These peaks were not detected under DSI-GC × GC/TOF-MS due to its higher LOQ. From this experiment, we learned that the analytical method based on HRMS with 2 μL injection has better sensitivity than DSI-GC × GC/TOF-MS with 10 μL injection, independent of sample preparation.

3.3. Optimization of GPC and further clean-up

Dioxins and PCBs are lipophilic, so these compounds are mostly accumulated in fat. Previous studies show that significantly high levels of dioxins and PCBs are detected in fish or fish oil [25,29–31], but due to potential health benefits from fatty acids in fish, consumption of fish oil supplements is increasing. Due to possible contamination of the fish, screening of dioxins and PCBs could be a wise precaution before the fish oil is marketed.

To separate the 17 PCDD/Fs and 4 non-ortho PCBs from the bulk matrix, we employed an automated GPC installed with an Express performance column (24 g of BioBeads S-X3, 2 cm i.d. and 22.5 cm length). The size of the column is 30% smaller than the traditional GPC column called for in the EPA 1613 method. This Express column reduced running time and solvent consumption considerably. Elution profiles of different amounts of cod liver oil, dioxins, and PCBs were tested to maximize the sample size, and the maximum amount of injected cod liver oil was determined to be 2 g (see Fig. 3) due to overloading of the column and consequent tailing of the lipid peak. Based on the elution profiles (Fig. 3), the collection time was chosen from 12.5 min to 17.5 min at flow rate 5 mL/min (~25 mL).

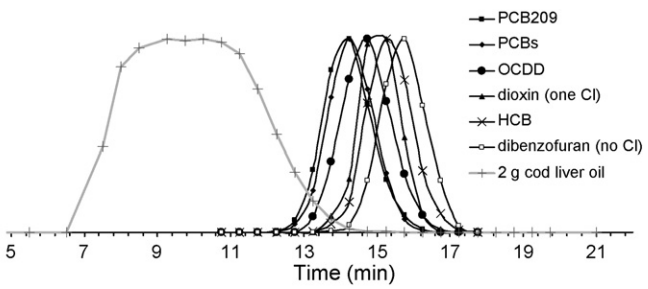


Fig. 3. Elution profiles for 5 mL injection containing 2 g cod liver oil and various compounds in GPC (5 mL/min flow rate).

After GPC clean-up, 10–50 mg of lipid material still remained in the collected fraction. Because the final extract volume prior to DSI was ≤100 μL, 10–50 mg of co-extracted material was expected to exceed the capabilities of DSI and reduce transfer efficiency of the analytes, thus further clean-up was still necessary.

Previous studies involving pesticide residue analysis showed that a combination of PSA, C_{18}, and anhydrous $MgSO_4$ in dispersive-SPE was effective for removal of fatty acids and other lipids from food matrices [23,33]. Due to sample size limitations in this case for analysis of dioxins, we chose not to use the dispersive-SPE format and tested cartridge-type SPE instead. However, neither PSA nor C_{18} nor their combination were effective in removing the co-extracted components from the 25 mL fish oil fractions in 1:1 EtOAc:cyclohexane. The solvent was much more lipophilic than the acetonitrile used previously, which is the most likely reason why this SPE clean-up was unsuccessful.

Re-injection of the collected fraction in GPC after evaporating the solvent provided significant removal of the co-eluting lipids from the first injection. This twice-injected GPC extract was evaporated to dryness and then spiked with 100 μL of the 17 PCDD/F standard solution in iso-octane (TCDD at 2.5 pg/μL). In DSI-GC × GC/TOF-MS, significant interferences of the molecular ions still occurred for the smaller congeners, such as TCDF, TCDD, and non-ortho PCB 77 and 81. For example, Fig. 4(A) shows three 2D slices of *m*/*z* 322 and 320 ions. Among them, the second and third slices include TCDD, but it is clear that there are significant interferences. The interferences almost co-eluted with each of the TCDD 2D slices so that the automatic peak finding function in the data analysis software could not integrate the analyte peaks. Even manual

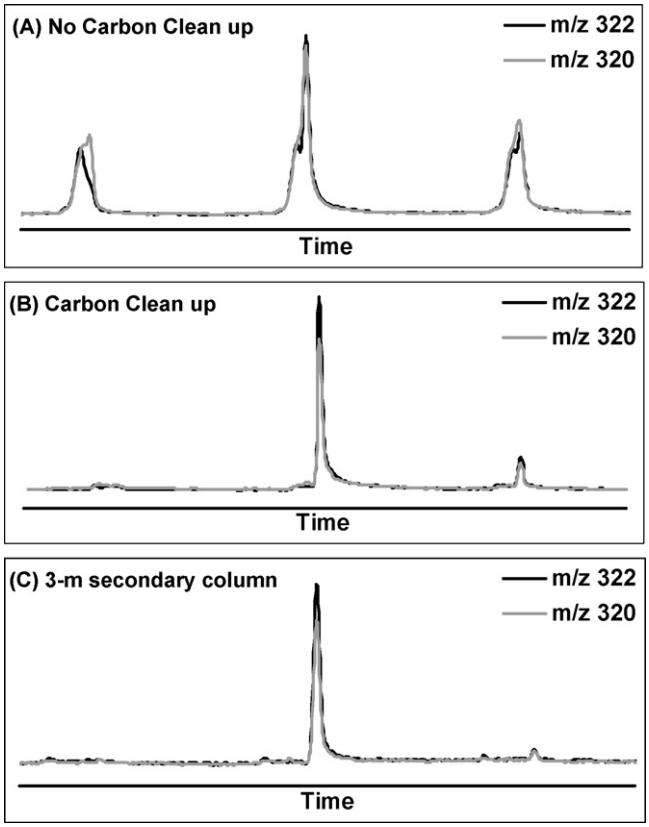


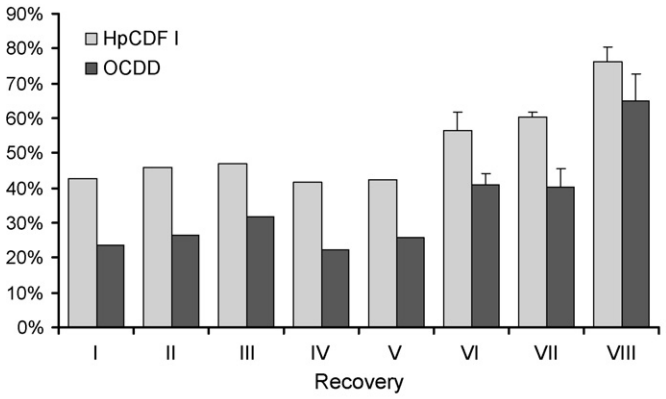
Fig. 4. TCDD slices and co-eluting interferences at *m*/*z* 320 and 322 in GC × GC for cod liver oil extract spiked with PCDD/Fs (125 pg/g TCDD) after: (A) two sequential GPC clean-ups; (B) one GPC clean-up followed by a clean-up with GCB in reversible-SPE; and (C) after the same clean-up as in (B) but using a longer secondary column (3-m instead of 2-m).

integration did not work well, and integration error was too great, especially at low concentrations. Since the analysis for dioxins and non-ortho PCBs required lower LOQs, these interferences had to be removed. Based on their mass spectra, the interferences contained several long-chain hydrocarbons, including high intensities of m/z 79, 91, 105, 107, 117, 131, etc. Interfering matrix peaks with TCDF also had similar mass spectra, which suggested that the interferences eluted in a broad peak from the 1D GC column and were chopped into many slices in 2D GC × GC operation. This was confirmed by evaluating 1D GC chromatograms of the injected sample.

To remove the interference, various commercially available SPE sorbents, such as PSA, C_{18}, silica, alumina, and GCB, were evaluated by loading the entire extract (25 mL of EtOAc:cyclohexane) from the second (re-injected) GPC clean-up. Among them, SPE with GCB was the most efficient to separate the analytes from the interferences, and it worked well enough to eliminate the second GPC step altogether. GCB strongly retains structurally planar molecules, such as PCDD/Fs and non-ortho PCBs, and back-flushing with toluene was needed to elute the analytes in a reasonable volume (benzene was avoided due to safety concerns). Fig. 4(B) shows how the interferences were reduced and the TCDD slices are clearly visible (this was also observed for the other analytes). However, there were still very small intensities of interferences prior to the TCDD slices in Fig. 4(B). These interferences caused errors in integration of the analytes at low concentrations. However, these interfering slices were also detected in procedural blanks, which suggested that the interferences originated from the SPE step, presumably from the polypropylene cartridges. These interferences were clearly separated by simply using a longer 2D column (3-m instead of 2-m [24]), as shown in Fig. 4(C).

A downside of using SPE with GCB for clean-up was that toluene has a higher boiling point of 110.6 °C vs. iso-octane (99.3 °C). Thus, evaporation of toluene required longer time and higher temperature. To reduce toluene consumption and evaporation time, we evaluated various conditions (loading volumes, rinsing solvent type, and two different types of GCB). Because high recoveries of the smaller PCDD/Fs and 4 non-ortho PCBs were observed with 5 mL toluene, we focused on the recoveries of the internal standards, $^{13}C_{12}$-labeled HpCDF I and OCDD (see Fig. 5). Commercially available Supelclean Envi-Carb custom-packed SPE (175 mg in a 0.5 mL polypropylene reversible tube) was tested at six conditions (I–VI), as shown in Fig. 5. The recoveries of $^{13}C_{12}$-HpCDF I and $^{13}C_{12}$-OCDD under conditions I–V are only around 25–40%. There was no difference to use warm toluene (50 °C), and in the cases of conditions III–V, non-ortho PCBs, TCDD, and TCDF were eluted with a wash solvent in reverse flow (DCM or combination of DCM/hexane). We observed higher recovery when 1 mL of 1:1 EtOAc:cyclohexane was loaded onto the SPE cartridge (condition VI) instead of 25 mL. This suggested that loading with a larger volume of solvent pushed the analytes farther down the GCB column. Therefore, condition VI was an option but it required a solvent evaporation step prior to loading to SPE. When we tested a different type of GCB sorbent, Hypersep Hypercarb, the maximum amount that could be packed in the same volume of reversible SPE cartridge was 100 mg due to its lower density (higher porosity). A higher recovery was achieved for the 100 mg Hypercarb than the 175 mg Envi-Carb GCB. In an additional experiment, 100 mg of Supelclean Envi-Carb was tested, and higher recovery was consistently observed when Hypersep Hypercarb was utilized.

Toluene is not a good solvent for the DSI system because of its high boiling point, which would require higher temperature and longer evaporation time and likely cause a loss of analytes during venting. We needed to concentrate the extract in any event, so we chose to conduct a solvent-exchange from toluene to iso-octane for analysis. In a simple experiment, we confirmed that the recoveries



I: 25 mL of 1:1 cyclohexane:EtOAc spiked with standards loaded to SPE (175 mg of Enviro-Carb)
II: Same as I, but warm toluene (50°C) used
III: Same as I, but 5 mL of DCM loaded to SPE in reverse flow prior to toluene
IV: Same as III, but 50% of DCM in hexane used instead of 5 mL of DCM
V: Same as III, but 10% of DCM in hexane used instead of 5 mL of DCM
VI: Same as I, but 1 mL of 1:1 cyclohexane:EtOAc spiked with standards loaded to SPE (n = 3)
VII: Same as I, but 100 mg of Enviro-Carb used (n = 3)
VIII: Same as I, but 100 mg of Hyper-Carb used (n = 3)

Fig. 5. Comparison of recoveries of $^{13}C_{12}$-HpCDF I and $^{13}C_{12}$-OCDD with 5 mL of toluene elution solvent in various tests of the reversible-SPE clean-up step with GCB. I: 25 mL of 1:1 cyclohexane:EtOAc spiked with standards loaded to SPE (175 mg of Enviro-Carb); II: same as I, but warm toluene (50 °C) used; III: same as I, but 5 mL of DCM loaded to SPE in reverse flow prior to toluene; IV: same as III, but 50% of DCM in hexane used instead of 5 mL of DCM; V: same as III, but 10% of DCM in hexane used instead of 5 mL of DCM; VI: same as I, but 1 mL of 1:1 cyclohexane:EtOAc spiked with standards loaded to SPE (n = 3); VII: same as I, but 100 mg of Enviro-Carb used (n = 3); VIII: same as I, but 100 mg of Hyper-Carb used (n = 3).

of the internal standards (4 non-ortho $^{13}C_{12}$-PCBs and five $^{13}C_{12}$-PCDD/Fs) were acceptable (80–100%) during the solvent exchange step.

3.4. Method validation

Once the final sample preparation procedure was set, matrix-matched calibration standards were prepared at five different levels. For example, TCDD was spiked in the final cod liver oil extract equivalent to 5 pg/g, 10 pg/g, 25 pg/g, 50 pg/g, and 100 pg/g, and the other congeners' levels followed the ratios listed in Table 3. Strong linear regression was achieved for all of the analytes (R^2 values ranged from 0.940–0.999). Fig. 6 shows the detection of 1 pg TCDD with S/N = 23 for 10 μL injection using DSI from the 100 μL matrix-matched calibration standard solution (0.1 pg/μL, equivalent to 5 pg/g in the original sample). Following the relationship in which LOQ has S/N = 10, the LOQ in this case for TCDD was 2.1 pg/g (for

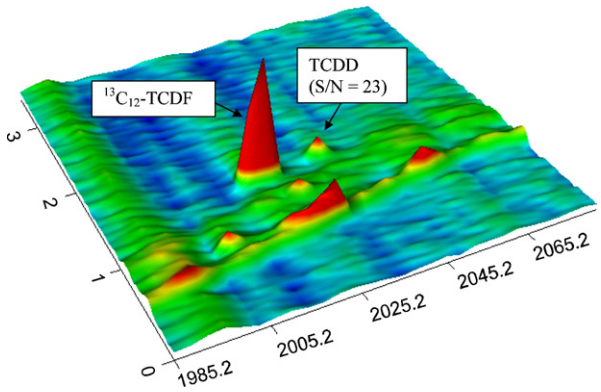


Fig. 6. DSI-GC × GC/TOF-MS chromatogram of 1 pg TCDD (m/z 322) injected at 0.1 pg/μL (5 pg/g cod liver oil equivalent) in a matrix-matched solution.

Table 3
Codes, toxic equivalent factors (TEFs), LOQs, and average %recoveries (standard deviations) of the 17 PCDD/Fs and 4 non-ortho PCBs obtained with the DSI-GC × GC/TOF-MS method for spiked cod liver oil samples (n = 3 at each level)

Compound	Code	TEF [42]	LOQ	LOQ (TEQ)	Ratio[a]	5 pg/g	50 pg/g	100 pg/g
Polychlorinated dibenzo-*p*-dioxins								
2,3,7,8-TeCDD	TCDD	1	2.1	2.1	1	101 (29)	108 (4.0)	106 (7.4)
1,2,3,7,8-PeCDD	PCDD	1	7.8	7.8	2.5	110 (1.6)	130 (13)	125 (0.92)
1,2,3,4,7,8-HxCDD	HxCDD I	0.1	13	1.3	2.5	<LOQ	119 (13)	102 (15)
1,2,3,6,7,8-HxCDD	HxCDD II	0.1	10	1.0	2.5	[b]	104 (20)	121 (11)
1,2,3,7,8,9-HxCDD	HxCDD III	0.1	16	1.6	2.5	<LOQ	86 (13)	94 (4.4)
1,2,3,4,6,7,8-HpCDD	HpCDD	0.01	60	0.60	2.5	<LOQ	112 (1.7)	104 (8.9)
OCDD	OCDD	0.0003	190	0.057	5	<LOQ	100 (51)	102 (7.2)
Polychlorinated dibenzofurans								
2,3,7,8-TeCDF	TCDF	0.1	0.94	0.094	1	86 (4.2)	102 (5.6)	107 (5.5)
1,2,3,7,8-PeCDF	PCDF I	0.03	2.2	0.066	2.5	103 (17)	127 (6.5)	131 (8.1)
2,3,4,7,8-PeCDF	PCDF II	0.3	3.1	0.93	2.5	102 (22)	108 (7.5)	107 (6.5)
1,2,3,4,7,8-HxCDF	HxCDF I	0.1	5.2	0.52	2.5	85 (20)	113 (22)	105 (4.5)
1,2,3,6,7,8-HxCDF	HxCDF II	0.1	5.8	0.58	2.5	72 (6.7)	100 (6.2)	106 (8.6)
2,3,4,6,7,8-HxCDF	HxCDF III	0.1	7.0	0.70	2.5	[b]	77 (14)	73 (7.3)
1,2,3,7,8,9-HxCDF	HxCDF IV	0.1	13	1.3	2.5	<LOQ	98 (16)	111 (9.2)
1,2,3,4,6,7,8-HpCDF	HpCDF I	0.01	19	0.19	2.5	<LOQ	112 (7.7)	108 (0.91)
1,2,3,4,7,8,9-HpCDF	HpCDF II	0.01	27	0.27	2.5	<LOQ	134 (17)	121 (9.7)
OCDF	OCDF	0.0003	120	0.036	5	<LOQ	148 (20)	199 (35)
Polychlorinated biphenyls (non-ortho)						7 pg/g	35 pg/g	70 pg/g
3,3′,4,4′-TeCB	CB77	0.0001	0.79	0.000079	1	66 (8.0)	106 (4.3)	108 (3.0)
3,4,4′,5-TeCB	CB81	0.0003	1.1	0.00033	1	123 (5.3)	114 (4.9)	113 (3.2)
3,3′,4,4′,5-PeCB	CB126	0.1	2.3	0.23	1	127 (17)	109 (6.8)	99 (8.3)
3,3′,4,4′,5,5′-HxCB	CB169	0.03	2.1	0.063	1	[b]	99 (16)	104 (4.9)

[a] Spiked amount of each PCDD/F congener ratio to TCDD and each PCB congener to CB77.
[b] Not detected.

100% recovery). The consistency of the DSI-GC × GC/TOF-MS analysis was remarkable over time, and although the LOQ is typically an estimation, the analytical method achieved this LOQ reproducibly in repetitive experiments.

For determination of recoveries and repeatabilities, three replicates of cod liver oil fortified at each of three levels were prepared (5, 50, and 100 pg/g of TCDD and 7, 35, and 70 pg/g of non-ortho PCBs, with other PCDD/F congeners following the ratios given in Table 3). Recoveries of all analytes fell between 66 and 131% except OCDF. Some analytes could not be quantified because the lowest spiking levels were below their LOQs, and two others were not detected at the lowest level.

In the method validation experiment, we noticed some differences in the amount of non-volatiles left in the microvials among the samples. These non-volatiles in the microvials from the samples either remained as a large single drop after injection or many fine droplets on the wall of the microvials. The non-volatiles in the microvials in both cases must have a planar structure because they were retained by the GCB (Hypercarb). We believe that the non-volatiles consisted mainly of calciferols (vitamin D), which have a planar structure, and cod liver oil is known to be one of the richest sources of vitamin D [34]. The vitamin D probably caused lower transfer of the least volatile analytes from the microvial to the GC column in DSI, and variable amounts of vitamin D in the microvials in samples and matrix-matched calibration standards affected recoveries of heavy molecules, such as OCDF. Non-volatile content is known to affect thermo-desorption of larger molecules more negatively, which was shown previously in injection of oil solutions [35]. However, a major advantage of the DSI approach is that the non-volatiles did not build up in the liner or contaminate the GC system. Through the use of DSI, less clean-up of extracts was needed and routine maintenance of the system was reduced. We chose to sacrifice better results for the least important analytes, OCDD and OCDF, by reducing the elution volume of toluene during reversible-SPE and use of DSI in the presence of vitamin D.

3.5. Applicability of the method

The World Health Organization (WHO) has set a tolerable daily intake (TDI) of 1–4 pg toxic equivalency quotients (TEQs)/kg body weight per day [36]. The European Union (EU) set maximum levels (MLs) for PCDD/Fs in various food products, and the ML for fish oil was set at 2 pg/g TEQ and 10 pg/g TEQ as a combined sum of the PCDD/Fs alone and including dioxin-like PCBs, respectively [29,37]. Considering these MLs, the LOQs directly and LOQs in terms of TEQs in the DSI-GC × GC/TOF-MS method are too high to detect all of the analytes at the MLs. For instance, the method is not sensitive enough to measure TCDD and PeCDD (the most toxic congeners) at sufficiently low levels. Our primary objective was to develop a fast and easy analytical method, so we limited the size of the GPC column, which allowed a maximum of 2 g of cod liver oil to be injected. If we increased the sample size up to 10 g of cod liver oil with a larger column or multiple injections of 2 g portions, the LOQs would be significantly lowered. In an experiment, we tested 2 g, 4 g and 8 g of cod liver oil samples (multiple 2 g injections in GPC were made due to the small column), and each final extract was spiked with the same standard PCDD/F solution and analyzed by DSI-GC × GC/TOF-MS. We confirmed that the three GC chromatograms were almost identical, and interferences were no different for the larger sample sizes.

However, sample preparation of a larger amount of cod liver oil (or other fish oil) requires a bigger GPC column, more solvent consumption, and longer sample preparation time. Therefore, we sought for a way to use our method without increasing sample size. There are several studies to assess dioxin levels in fish tissue or fish oil. According to the data from the literature and a recently conducted FAPAS proficiency test for PCBs and dioxins in cod liver oil, four congeners (TCDF, TCDD, PCDF II, and PCDD) contributed (≥90%) to the total TEQ among the 17 PCDD/Fs in all of the samples [30–32,38–40]. Among these congeners, TCDF had the lowest LOQ (TEQ) of 0.094 pg/g in our method. In other studies, the mean contribution of TCDF (TEF = 0.1) to the total TEQ was 18% (n = 15,

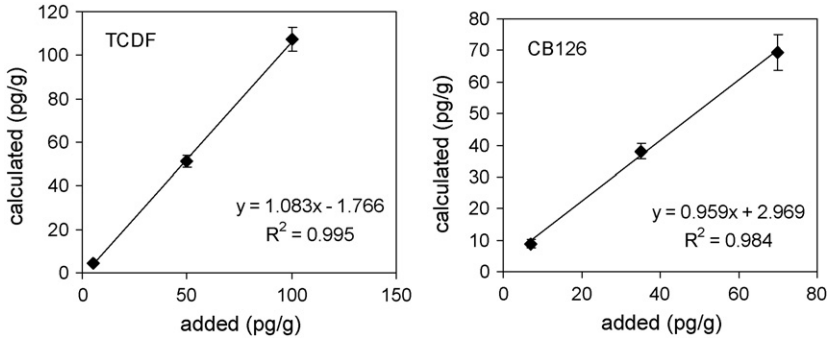


Fig. 7. Method validation: measurement of 5 pg/g, 50 pg/g, and 100 pg/g of TCDF and 7 pg/g, 35 pg/g, 70 pg/g of CB126 spiked in cod liver oil samples ($n = 3$).

lowest 7% and highest 49%) [30–32,38–40]. If we apply the lowest contribution of 7% for the TDI ML of 2 pg/g (TEQ) for fish oil, TCDF constitutes 0.14 pg/g TEQ corresponding to 1.4 pg/g, which is still above its LOQ in our method. Therefore, we suggest that if TCDF is quantified above its LOQ in our method, the sample likely exceeds the TDI ML.

The real data monitoring fish and fish oil also show that CB126 (TEF = 0.1) is the largest contributor to a combined total TEQ of the 17 PCDD/Fs and dioxin-like PCBs (4 non-ortho PCBs, and mono-ortho PCBs) [29–32,39]. Its contribution range was 43–68% [30–32,40], and Fernandes et al. found that the average non-ortho PCB contribution to the total TEQ was 55% in their extensive study for 33 fish oil supplements purchased during 2000–2001 [29]. Considering that CB126 contributes ≥95% to the TEQ of non-ortho PCBs, we can assume that CB126 constitutes the most important PCB to be monitored. According to the monitoring data, the mean contribution of CB126 was 46% to the total TEQ, and its range varied from 13 to 77% [29]. If we take the lowest value of 13% as the CB126 contribution to the total TEQ, and for 10 pg/g TEQ (EU's ML), a CB126 TEQ concentration of 1.3 pg/g would correspond to 13 pg/g of actual CB126 concentration. This level is higher than its LOQ of 2.3 pg/g in our method.

Therefore, we can reasonably use TCDF and CB126 as markers for screening dioxin and dioxin-like PCBs in fish or fish oil samples. Fig. 7 presents the strong linear relationships for TCDF and CB126 at three different spiking levels with good agreement between the spiked levels and the calculated levels. For rapid screening of dioxins in foodstuffs, measurement of PCBs (PCB indicators) is often used to signify dioxin contamination because higher PCBs levels tend to correspond to higher total TEQs. However, this is only the case if PCBs are the source of dioxin contamination [41–44]. Therefore, screening only PCBs can overlook non-PCB sources of dioxin contamination, and our method would be more beneficial in those cases to monitor dioxin and dioxin-like compounds in foodstuff.

4. Conclusions

In this study, the operating parameters of DSI for the analysis of 17 PCDD/Fs were optimized, and a more streamlined sample preparation method was developed. For the optimization of DSI parameters, statistical design of experiments was utilized to quickly screen which parameters were most significant. The optimized DSI-GC × GC/TOF-MS performance was demonstrated to be acceptable in side-by-side comparison with HRGC-HRMS of shared standards. For sample preparation of cod liver oil, automated GPC was utilized to isolate the 17 PCDD/Fs and 4 non-ortho PCBs from the lipid matrix. The maximum sample size of cod liver oil for GPC injection was 2 g based on the elution profiles of the cod liver oil. For further clean-up to remove interferences from matrices, reversible-SPE with GCB was employed, and Hypercarb was found to work better than Envi-Carb for reducing the amount of toluene used for elution of the dioxins. Acceptable recoveries for most analytes at three different levels were achieved in the method validation check of our final analytical method, but the LOQs of the analytes were too high to meet the needs for congener-specific dioxin analysis at current regulatory levels. However, based on the literature about dioxin and dioxin-like PCBs in fish and fish oil samples, our method can be used for analytical screening of a large number of fish oil (or fish) samples using TCDF and CB126 as markers. The LOQs of TCDF and CB126 were 0.94 pg/g and 2.3 pg/g, respectively, which were below the critical concentrations of 1.4 pg/g and 13 pg/g, respectively, calculated from the literature data and TDI MLs. The entire procedure takes about 2 h per sample (25 min for GPC, 30 min for SPE clean-up and solvent exchange, and 63 min for DSI-GC × GC/TOF-MS), or a batch of 15 samples can be prepared in an 8 h day for an overnight analysis. Full automation of this method is possible, and the fact that sample preparation takes the same amount of time as the analysis lends the method to parallel processing for maximum sample throughput. Greatly reduced maintenance of the instrument is another major advantage of the DSI approach.

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